REPORT

Study Title

EVALUATION OF THE MUTAGENIC ACTIVITY OF [redacted] IN THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY AND THE *ESCHERICHIA COLI* REVERSE MUTATION ASSAY (WITH INDEPENDENT REPEAT)

Author

[redacted]

Study completion date

09 October 2006

Test Facility

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Laboratory Project Identification

NOTOX Project [redacted]
NOTOX Substance [redacted]

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2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

Analysis of stability, homogeneity and concentration of the test substance under test conditions was not performed as part of this study. Information concerning stability of the test substance in vehicle was available.

NOTOX B.V.

[redacted]
Study Director

[redacted]
Head of In Vitro & Environmental Toxicology

[redacted]

Date: 09 October 2006 Date: 10/10/2006

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

The reporting date is the date of reporting to the Study Director. The QAU report was then forwarded to the Test Facility Management.

Type of inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Protocol	21-Aug-06	21-Aug-06	21-Aug-06
	Amendment 1 of protocol	14-Sep-06	14-Sep-06	14-Sep-06
	Report	21-Sep-06	21-Sep-06	21-Sep-06
Process	**Genetic and In Vitro Toxicology**	18-Jul-06	21-Jul-06	21-Jul-06
	Test substance handling			
	Exposure			
	Observations/Measurements			
	Specimen handling			

Head of Quality Assurance
[redacted]

[redacted]

Date: [redacted]

4. SUMMARY

Evaluation of the mutagenic activity of [redacted] in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat).

[redacted] was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* (WP_2uvrA). The test was performed in two independent experiments in the presence and absence of S9-mix (rat liver S9-mix induced by a combination of phenobarbital and ß-naphthoflavone). To obtain more information about the possible mutagenicity of [redacted] an additional experiment was performed with the strains TA1535, TA1537 and TA98 in the absence and presence of S9-mix.

The study procedures described in this report were based on the most recent OECD and EEC guidelines.

Batch [redacted] of [redacted] was a dark purple powder with a purity of >95%. The test substance was dissolved in dimethyl sulfoxide.

In the dose range finding test, [redacted] was tested up to concentrations of 5000 µg/plate in the absence and presence of S9-mix in the strains TA100 and WP_2uvrA. [redacted] precipitated on the plates at the dose level of 5000 µg/plate. Cytotoxicity, as evidenced by a reduction of the bacterial background lawn, was only observed in tester strain TA100 in the absence and presence of S9-mix at the dose levels of 3330 and 5000 µg/plate.

Based on the results of the dose range finding test, [redacted] was tested in the first mutation assay at a concentration range of 10 to 1000 µg/plate in the absence and presence of 5% (v/v) S9-mix in tester strains TA1535, TA1537 and TA98. [redacted] did not precipitate on the plates at the dose level of 1000 µg/plate. The bacterial background lawn was not reduced at any of the concentrations tested and no decrease in the number of revertants was observed.

Since in the first mutation assay no dose level with toxicity or precipitate on the plates was tested, an additional experiment was performed with tester strains TA1535, TA1537 and TA98. [redacted] was tested in the second mutation assay at a concentration range of 1000 to 5000 µg/plate in the absence and presence of 5% (v/v) S9-mix. [redacted] precipitated on the plates at the top dose of 5000 µg/plate. Toxicity was observed in all tester strains, except in the tester strain TA98 in the presence of S9-mix.

In an independent repeat of the assay with additional parameters, [redacted] was tested at a concentration range of 33 to 5000 µg/plate in the absence and presence of 10% (v/v) S9-mix in tester strains TA1535, TA1537, TA98, TA100 and WP_2uvrA. [redacted] precipitated on the plates at the top dose of 5000 µg/plate. Toxicity was observed in all tester strains, except in the tester strains TA98 in the presence of S9-mix and WP_2uvrA in the absence and presence of S9-mix.

[redacted] did not induce a significant dose-related increase in the number of revertant (His^+) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp^+) colonies in tester strain WP_2uvrA both in the absence and presence of S9-metabolic activation. These results were confirmed in independently repeated experiments.

In this study, the negative and strain-specific positive control values were within our laboratory historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that [redacted] is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

5. INTRODUCTION

5.1. Preface

Sponsor	[redacted]
Study Monitor	[redacted]
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	[redacted]
Technical Coordinator	[redacted]
Study Plan	Start : 30 August 2006 Completion : 18 September 2006

5.2. Aims of the study

The objective of this study was to evaluate the test substance for its ability to induce reverse mutations in a gene of histidine-requiring *Salmonella typhimurium* bacterial strains resulting in histidine-independent strains, and in a gene of tryptophan-requiring *Escherichia coli* bacterial strain resulting in a tryptophan-independent strain.

Background of the test system
The *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay have been shown to be rapid and adequate indicators for the mutagenic activity of a wide range of chemical compounds.

The assay was conducted in the absence and presence of a metabolizing system (S9-mix).

The *Salmonella typhimurium* strains used in this study were TA1535, TA1537, TA98 and TA100. The *Escherichia coli* strain used was WP_2uvrA. The strains TA1537 and TA98 are capable of detecting frameshift mutagens, strains TA1535, TA100 and WP_2uvrA are capable of detecting base-pair substitution mutagens (Ref. 1, 2, 3, 4 and 5).

5.3. Guidelines

The study procedures described in this report were based on the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals; Guideline no. 471: "Genetic Toxicology: Bacterial Reverse Mutation Test" (Adopted July 21, 1997).
- European Economic Community (EEC). Directive 2000/32/EC, Part B: Methods for the Determination of Toxicity; B.13/14: "Mutagenicity: "Reverse Mutation Test using bacteria". EEC Publication Commission Directive (Published June 8, 2000).

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization

of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

6. MATERIALS AND METHODS

6.1. Test substance

6.1.1. Test substance information

Identification	[redacted]
Structure	
Molecular formula	[redacted]
Molecular weight	1317.45
Description	Dark purple powder
Batch	[redacted]
Purity	>95% (NMR)
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	01 January 2008

6.1.2. Study specific test substance information

Stability at higher temperatures	Maximum temperature 75°C, maximum duration 48 hours
Stability in vehicle	Dimethyl sulfoxide: At least 96 hours
Solubility in vehicle	Dimethyl sulfoxide: Yes

6.1.3. Test substance preparation

The test substance was dissolved in dimethyl sulfoxide of spectroscopic quality (Uvasol, Merck, Darmstadt, Germany). Test substance concentrations were used within 4 hours after preparation.

6.2. Reference substances

6.2.1. Negative control

The vehicle of the test article, being dimethyl sulfoxide.

6.2.2. Positive controls

Without metabolic activation (-S9-mix):

Strain	Chemical	Concentration/plate	Solvent
TA1535	sodium azide (SA) (Sigma, Zwijndrecht, The Netherlands)	5 µg	Saline
TA1537	9-aminoacridine (9AC) (Acros Organics, Geel, Belgium)	60 µg	Milli-Q water
TA98	2-nitrofluorene (NF) (Merck)	10 µg	DMSO
TA100	methylmethanesulfonate (MMS) (Sigma)	650 µg	DMSO
WP_2uvrA	4-nitroquinoline N-oxide (4-NQO) (Sigma)	10 µg	DMSO

With metabolic activation (+S9-mix):
The positive control substance used for all tester strains was 2-aminoanthracene (2AA) (Sigma).
The following doses were used:

Strain	Concentration/plate	Amount of S9-mix	Solvent
TA1535	1 µg	5 and 10%	DMSO
TA1537	2.5 µg	5%	DMSO
TA1537	5 µg	10%	DMSO
TA98	1 µg	5 and 10%	DMSO
TA100	1 µg	5%	DMSO
TA100	2.5 µg	10%	DMSO
WP_2uvrA	10 µg	5 and 10%	DMSO

Solvents for reference substances
Saline = physiological saline (B. Braun, Melsungen AG, Germany)
DMSO = dimethyl sulfoxide of spectroscopic quality (Merck)
Milli-Q water (Millipore Corp., Bedford, MA., USA)

6.3. Test system

Test system	*Salmonella typhimurium* bacteria and *Escherichia coli* bacteria
Rationale	Recommended test system in international guidelines (e.g. OECD and EEC).
Source	*Salmonella typhimurium* strains: Dr. Bruce N. Ames, University of California at Berkeley, U.S.A. TA98 received on 21-02-1991, used batch: TA98.280406 TA1535 received on 30-07-2001, used batch: TA1535.190506 TA1537 received on 30-07-2001, used batch: TA1537.280406 Xenometric, Boulder, Co, U.S.A. (obtained from N.V. Organon) TA100 received on 19-09-2002, used batch: TA100.111105 *Escherichia coli* strain: Prof. Dr. B.A. Bridges, University of Sussex, Brighton, U.K. WP_2uvrA received on 23-10-1987, used batch: EC.190506

The characteristics of the different *Salmonella typhimurium* strains were as follows:

Strain	Histidine mutation	Mutation type
TA1537	*his*C3076	Frameshift
TA98	*his*D3052/R-factor*	Frameshift
TA1535	*his*G46	Base-pair substitutions
TA100	*his*G46/R-factor*	Base-pair substitutions

*: R-factor = plasmid pKM101 (increases error-prone DNA repair)

Each tester strain contained the following additional mutations:
rfa : deep rough (defective lipopolysaccharide cellcoat)
gal : mutation in the galactose metabolism
chl : mutation in nitrate reductase
bio : defective biotin synthesis
uvrB : loss of the excision repair system (deletion of the ultraviolet-repair B gene)

The *Salmonella typhimurium* strains were regularly checked to confirm their histidine-requirement, crystal violet sensitivity, ampicillin resistance (TA98 and TA100), UV-sensitivity and the number of spontaneous revertants.

The *Escherichia coli* WP_2uvrA strain detects base-pair substitutions. The strain lacks an excision repair system and is sensitive to agents such as UV. The sensitivity of the strain to a wide variety of mutagens has been enhanced by permeabilization of the strain using Tris-EDTA treatment (Ref.1). The strain was regularly checked to confirm the tryptophan-requirement, UV-sensitivity and the number of spontaneous revertants.

Stock cultures of the five strains were stored in liquid nitrogen (-196°C).

6.4. Cell culture

Preparation of bacterial cultures
Samples of frozen stock cultures of bacteria were transferred into enriched nutrient broth (Oxoid LTD, Hampshire, England) and incubated in a shaking incubator (37°C, 150 spm), until the cultures reached an optical density of 1.0 ± 0.1 at 700 nm (10^9 cells/ml). Freshly grown cultures of each strain were used for a test.

Agar plates
Agar plates (ø 9 cm) contained 25 ml glucose agar medium. Glucose agar medium contained per liter: 18 g purified agar (Oxoid LTD) in Vogel-Bonner Medium E, 20 g glucose (B. Braun, Melsungen, Germany). The agar plates for the test with the *Salmonella typhimurium* strains also contained 12.5 µg/plate biotin (Merck) and 15 µg/plate histidine (Merck) and the agar plates for the test with the *Escherichia coli* strain contained 15 µg/plate tryptophan (Acros Organics).

Top agar
Milli-Q water containing 0.6% (w/v) bacteriological agar (Oxoid LTD) and 0.5% (w/v) Sodium Chloride (Merck) was heated to dissolve the agar. Samples of 3 ml top agar were transferred into 10 ml glass tubes with metal caps. Top agar tubes were autoclaved for 20 min at 121 ± 3°C.

Environmental conditions
All incubations were carried out in the dark at 35.7 – 38.8°C (protocolled range 37.0 ± 1.0°C). Temporary deviations of maximally 1 hour (in the range of 34.0 – 38.5°C) occurred due to addition of plates (which were at room temperature) to the incubator or due to opening and closing the incubator door. Based on laboratory historical data these deviations are considered not to affect the study integrity.

6.5. Metabolic activation system

Rat liver microsomal enzymes were routinely prepared from adult male Wistar rats, which were obtained from Charles River, Sulzfeld, Germany.

6.5.1. Preparation of S9-fraction

The animals were housed at NOTOX in a special room under standard laboratory conditions, as described in the Standard Operating Procedures. The rats were orally dosed for three consecutive days with a suspension of phenobarbital (80 mg/kg body weight) and

ß-naphthoflavone (100 mg/kg body weight) in corn oil (they were denied access to food for 3 to 4 hours preceding each dosing). One day after the final exposure (24 h), the rats were sedated using oxygen/carbon dioxide and then killed by decapitation. The rats received a limited quantity of food during the night before sacrifice. The livers of the rats were removed aseptically, and washed in cold (0°C) sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Na_2-EDTA. Subsequently the livers were minced in a blender and homogenized in 3 volumes of phosphate buffer with a Potter homogenizer. The homogenate was centrifuged for 15 min at 9000 g. The supernatant (S9) was transferred into sterile ampules, which were stored in liquid nitrogen (-196°C) for a maximum of 1 year.

Before use, all S9-batches were characterized with the metabolic activation requiring positive control; benzo[a]pyrene (Sigma) in tester strain TA98 at the concentration of 5 µg/plate.

6.5.2. Preparation of S9-mix

S9-mix was prepared immediately before use and kept on ice. S9-mix components contained per 10 ml: 30 mg NADP (Randox) and 15.2 mg glucose-6-phosphate (Roche Diagnostics, Mannheim, Germany) in 5.5 ml Milli-Q water (first and second experiment) or 5.0 ml Milli-Q water (third experiment); 2 ml 0.5 M sodium phosphate buffer pH 7.4; 1 ml 0.08 M $MgCl_2$ solution; 1 ml 0.33 M KCl solution. The above solution was filter (0.22 µm)-sterilized. To 9.5 ml of S9-mix components 0.5 ml S9-fraction was added (5% (v/v) S9-fraction) to complete the S9-mix in the first and second experiment and to 9.0 ml of S9-mix components 1.0 ml S9-fraction was added (10% (v/v) S9-fraction) to complete the S9-mix in the third experiment. The S9-batch used was no. 06-4.

6.6. Study design

6.6.1. Dose range finding test

Selection of an adequate range of doses was based on a dose range finding test with the strains TA100 and WP_2uvrA, both with and without S9-mix. Eight concentrations, 3, 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate were tested in triplicate. This dose range finding test was reported as a part of the first experiment of the mutation assay. The highest concentration of [redacted] used in the subsequent mutation assay was 5 mg/plate.

6.6.2. Mutation assay

At least five different doses (increasing with approximately half-log steps) of the test substance were tested in triplicate in each strain.

The test substance was tested both in the absence and presence of S9-mix in each strain, in two independent experiments. An additional experiment was performed with the strains TA1535, TA1537 and TA98 in the absence and presence of S9-mix.

Top agar in top agar tubes was molten and heated to 45°C. The following solutions were successively added to 3 ml molten top agar: 0.1 ml of a fresh bacterial culture (10^9 cells/ml) of one of the tester strains, 0.1 ml of a dilution of the test substance in dimethyl sulfoxide and either 0.5 ml S9-mix (in case of activation assays) or 0.5 ml 0.1 M phosphate buffer (in case of non-activation assays). The ingredients were mixed on a Vortex and the content of the top agar tube was poured onto a selective agar plate. After solidification of the top agar, the plates were inverted and incubated in the dark at 37.0 ± 1.0 °C for 48 h. After this period revertant colonies (histidine independent (His^+) for *Salmonella typhimurium* bacteria and tryptophan independent (Trp^+) for *Escherichia coli*) were counted.

6.6.3. Colony counting

The revertant colonies (histidine independent or tryptophan independent) were counted manually if less than 40 colonies per plate were present. If more than 40 colonies were present, these could be counted automatically with a Biocount 4000 Pro-S-colony counter. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually. The condition of the bacterial background lawn was evaluated, both macroscopically and microscopically by using a dissecting microscope.

6.7. Electronic data capture

Observations/measurements in the study were recorded electronically using the following programme: REES version 1.5 (REES scientific, Trenton, NJ, USA): Environmental monitoring.

6.8. Interpretation

6.8.1. Acceptability of the assay

A *Salmonella typhimurium* reverse mutation assay and/or *Escherichia coli* reverse mutation assay is considered acceptable if it meets the following criteria:

a) The negative control data (number of spontaneous revertants per plate) should be within the laboratory historical range for each tester strain.

Strain		Minimum value	Maximum value	Mean	± 3 x S.D.
TA1535	- S9-mix	4	26	12	± 13
	+ S9-mix	3	28	11	± 12
TA1537	- S9-mix	3	18	6	± 8
	+ S9-mix	3	21	6	± 9
TA98	- S9-mix	12	43	20	± 19
	+ S9-mix	12	51	25	± 21
TA100	- S9-mix	61	193	130	± 65
	+ S9-mix	62	186	121	± 68
WP_2uvrA	- S9-mix	4	30	13	± 16
	+ S9-mix	4	30	13	± 17

b) The positive control chemicals should produce responses in all tester strains, which are within the laboratory historical range documented for each positive control substance. Furthermore, the mean plate count should be at least three times the concurrent vehicle control group mean.

Strain		Minimum value	Maximum value	Mean	± 3 x S.D.
TA1535	- S9-mix	181	1923	1042	± 1160
	+ S9-mix	58	636	159	± 221
TA1537	- S9-mix	79	927	304	± 437
	+ S9-mix	58	787	262	± 364
TA98	- S9-mix	109	1794	795	± 1118
	+ S9-mix	147	1703	529	± 756
TA100	- S9-mix	452	1593	977	± 588
	+ S9-mix	223	2001	954	± 958
WP_2uvrA	- S9-mix	64	1406	636	± 626
	+ S9-mix	56	929	229	± 406

c) The selected dose range should include a clearly toxic concentration or should exhibit limited solubility as demonstrated by the preliminary toxicity range-finding test or should extend to 5 mg/plate.

6.8.2. Data evaluation and statistical procedures

No formal hypothesis testing was done.
A test substance is considered negative (not mutagenic) in the test if:
a) The total number of revertants in tester strain TA100 is not greater than two (2) times the concurrent control, and the total number of revertants in tester strains TA1535, TA1537, TA98 or WP_2uvrA is not greater than three (3) times the concurrent control.
b) The negative response should be reproducible in at least one independently repeated experiment.

A test substance is considered positive (mutagenic) in the test if:
a) The total number of revertants in tester strain TA100 is greater than two (2) times the concurrent control, or the total number of revertants in tester strains TA1535, TA1537, TA98 or WP_2uvrA is greater than three (3) times the concurrent control.
b) In case a positive response will be repeated, the positive response should be reproducible in at least one independently repeated experiment.

The preceding criteria were not absolute and other modifying factors might enter into the final evaluation decision.

6.9. List of deviations

6.9.1. List of protocol deviations

1. The temperature was above the protocolled range of 37.0 ± 1.0 °C for approximately 40 minutes in the first mutation experiment (with a maximum of 38.5°C) and for approximately 1 hour in the second mutation experiment (with a maximum of 38.8°C). Evaluation: These short termed deviations were observed within three hours after initiation of the test and were caused by adjustment of the temperature in the incubator after placing an amount of selective agar plates in the incubator. The negative control data (number of spontaneous revertants per plate) were within the laboratory historical range for each tester strain, therefore these short deviations of the temperature has no effect on the results of the study.

The study integrity was not adversely affected by the deviations.

6.9.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

7. RESULTS

7.1. Dose range finding test

[redacted] was tested in the tester strains TA100 and WP_2uvrA with concentrations of 3, 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate in the absence and presence of S9-mix.

This dose range finding test is reported as a part of the first experiment of the mutation test (Table 3). The individual data are presented in Appendix II.

Precipitate
Precipitation of [redacted] on the plates was observed at the start and at the end of the incubation period at the concentration of 5000 µg/plate.

Toxicity
To determine the toxicity of [redacted] the reduction of the bacterial background lawn, the increase in the size of the microcolonies and the reduction of the revertant colonies were examined. The definitions are stated in Appendix I.

In tester strain WP_2uvrA, no reduction of the bacterial background lawn and no biologically relevant decrease in the number of revertants were observed.

In tester strain TA100, no biologically relevant decrease in the number of revertants was observed. The bacterial background lawn was slightly reduced at the dose level of 3330 µg/plate and moderately reduced at 5000 µg/plate both in the absence and presence of S9-mix.

Mutagenicity
In the dose range finding test, no biologically relevant increase in the number of revertants was observed upon treatment with [redacted] under all conditions tested.

7.2. Mutation assay

7.2.1. Experiment 1

Based on the results of the dose range finding test, [redacted] was tested up to the dose level of 1000 µg/plate in the absence and presence of 5% (v/v) S9-mix with the *Salmonella typhimurium* strains, TA1535, TA1537 and TA98. The results are shown in Table 3, the individual data are presented in Appendix II.

Precipitate
Precipitation of [redacted] on the plates was not observed at the start or at the end of the incubation period.

Toxicity
In the first mutation experiment, there was no reduction in the bacterial background lawn and no biologically relevant decrease in the number of revertants at any of the concentrations tested in all tester strains in the absence and presence of S9-mix.

Mutagenicity
In the first mutation experiment, no increase in the number of revertants was observed upon treatment with [redacted] under all conditions tested.

7.2.2. Experiment 2

Since in the first mutation assay no dose level with toxicity or precipitate on the plates was tested, an additional experiment was performed with tester strains TA1535, TA1537 and TA98. In the second mutation experiment, [redacted] was tested up to the dose level of 5000 µg/plate in the absence and presence of 5% (v/v) S9-mix. The results are shown in Table 4, the individual data are presented in Appendix II.

Precipitate
Precipitation of [redacted] on the plates was observed at the start and at the end of the incubation period at the concentration of 5000 µg/plate.

Toxicity
The reduction of the bacterial background lawn and the reduction in the number of revertants are presented in Table 1 (For definitions see Appendix I).

Table 1 Toxicity of [redacted] in the second experiment

(Reduction of the bacterial background lawn and in the number of revertant colonies)

Strain	Without S9-mix			With S9-mix		
	Dose (µg/plate)	Bacterial background lawn	Revertant colonies	Dose (µg/plate)	Bacterial background lawn	Revertant colonies
TA1535	3330	slight	-[1]	3330	slight	-[1]
	5000	slight	-[1]	5000	moderate	-[1]
TA1537	3330	moderate	-[1]	3330	slight	-[1]
	5000	moderate	moderate	5000	moderate	-[1]
TA98	5000	slight	-[1]	5000	-[2]	-[1]

-[1] No reduction in the number of revertants
-[2] No reduction of the bacterial background lawn

All other concentrations, not mentioned here, showed no reduction of the bacterial background lawn and no biologically relevant reduction in the number of revertant colonies.

Mutagenicity
In the second mutation experiment, no increase in the number of revertants was observed upon treatment with [redacted] under all conditions tested.

7.2.3. Experiment 3

To obtain more information about the possible mutagenicity of [redacted] a third mutation experiment was performed in the absence of S9-mix and in the presence of 10% (v/v) S9-mix. Based on the results of the first and second mutation assay, [redacted] was tested up to the dose level of 5000 µg/plate in strains TA1535, TA1537, TA98, TA100 and WP_2uvrA. The results are shown in Table 5, the individual data are presented in Appendix II.

Precipitate
Precipitation of [redacted] on the plates was observed at the start and at the end of the incubation period at the concentration of 5000 µg/plate.

Toxicity
In tester strain WP_2uvrA, no reduction of the bacterial background lawn and no biologically relevant decrease in the number of revertants were observed.

The reduction of the bacterial background lawn and the reduction in the number of revertants in the other tester strains are presented in Table 2 (For definitions see Appendix I).

Table 2 Toxicity of [redacted] in the third experiment

(Reduction of the bacterial background lawn and in the number of revertant colonies)

Strain	Without S9-mix			With S9-mix		
	Dose (µg/plate)	Bacterial background lawn	Revertant colonies	Dose (µg/plate)	Bacterial background lawn	Revertant colonies
TA1535	3330	slight	-[1]	3330	slight	-[1]
	5000	slight	-[1]	5000	moderate	-[1]
TA1537	3330	moderate	-[1]	3330	moderate	-[1]
	5000	moderate	slight	5000	moderate	moderate
TA98	5000	slight	moderate	5000	-[2]	-[1]
TA100	3330	slight	-[1]	3330	slight	-[1]
	5000	moderate	-[1]	5000	moderate	-[1]

-[1] No reduction in the number of revertants
-[2] No reduction of the bacterial background lawn

All other concentrations, not mentioned here, showed no reduction of the bacterial background lawn and no biologically relevant reduction in the number of revertant colonies.

Mutagenicity
In the third mutation experiment, no increase in the number of revertants was observed upon treatment with [redacted] under all conditions tested.

8. DISCUSSION AND CONCLUSION

All bacterial strains showed negative responses over the entire dose range, i.e. no significant dose-related increase in the number of revertants in independently repeated experiments.

The negative and strain-specific positive control values were within our laboratory historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that [redacted] is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

9. REFERENCES

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Table 3 **Experiment 1: Mutagenic response of [redacted] in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay**

Day of performance:
TA100 and WP_2uvrA: 30 August 2006
TA1535, TA1537 and TA98: 08 September 2006

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain				
	TA1535	TA1537	TA98	TA100	WP_2uvrA
Without S9-mix					
positive control	1134 ± 36	325 ± 23	1130 ± 4	1335 ± 11	753 ± 37
solvent control	15 ± 3	10 ± 3	28 ± 3	178 ± 10	18 ± 5
3				185 ± 10	14 ± 1
10	15 ± 2	10 ± 3	32 ± 7	174 ± 3	12 ± 4
33	17 ± 3	9 ± 3	32 ± 4	189 ± 11	13 ± 3
100	19 ± 8	9 ± 1	30 ± 3	174 ± 4	10 ± 1
333	18 ± 3	9 ± 2	33 ± 3	196 ± 14	15 ± 3
1000	18 ± 2	9 ± 0	30 ± 4	194 ± 13	14 ± 6
3330				187 ± 6 s	12 ± 4
5000 SP				194 ± 30 m	18 ± 2
With S9-mix[1]					
positive control	207 ± 23	388 ± 40	695 ± 79	1273 ± 26	292 ± 15
solvent control	17 ± 5	8 ± 2	41 ± 4	180 ± 9	15 ± 4
3				181 ± 12	17 ± 3
10	14 ± 2	11 ± 5	32 ± 4	180 ± 19	16 ± 2
33	10 ± 2	7 ± 3	36 ± 6	189 ± 19	13 ± 3
100	19 ± 2	10 ± 2	33 ± 7	175 ± 16	14 ± 2
333	16 ± 3	10 ± 2	36 ± 5	199 ± 6	13 ± 2
1000	15 ± 3	7 ± 1	36 ± 3	201 ± 23	24 ± 1
3330				175 ± 16 s	15 ± 2
5000 SP				204 ± 20 m	18 ± 4

Solvent control: 0.1 ml dimethyl sulfoxide
1 The S9-mix contained 5% (v/v) S9 fraction
s Bacterial background lawn slightly reduced
m Bacterial background lawn moderately reduced
SP Slight Precipitate

Table 4 **Experiment 2: Mutagenic response of [redacted] in the *Salmonella typhimurium* reverse mutation assay**

Day of performance: 12 September 2006

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium*		
	TA1535	TA1537	TA98
Without S9-mix			
positive control	992 ± 18	568 ± 134	1125 ± 64
solvent control	10 ± 4	5 ± 3	25 ± 4
1000	9 ± 2	8 ± 3	21 ± 3
3330	7 ± 3 s	4 ± 1 m	19 ± 2
5000 SP	7 ± 2 s	2 ± 2 m	16 ± 6 s
With S9-mix[1]			
positive control	200 ± 16	382 ± 102	669 ± 5
solvent control	12 ± 3	10 ± 3	21 ± 7
1000	10 ± 3	6 ± 3	21 ± 3
3330	7 ± 2 s	5 ± 1 s	17 ± 3
5000 SP	9 ± 2 m	5 ± 2 m	16 ± 4

Solvent control: 0.1 ml dimethyl sulfoxide
1 The S9-mix contained 5% (v/v) S9 fraction
s Bacterial background lawn slightly reduced
m Bacterial background lawn moderately reduced
SP Slight Precipitate

Table 5 **Experiment 3: Mutagenic response of [redacted] in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay**

Day of performance: 15 September 2006

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain				
	TA1535	TA1537	TA98	TA100	WP_2uvrA
Without S9-mix					
positive control	1022 ± 23	229 ± 28	1116 ± 34	1474 ± 63	810 ± 30
solvent control	7 ± 3	3 ± 1	20 ± 5	117 ± 6	16 ± 4
33	7 ± 3	5 ± 2	25 ± 4	109 ± 5	18 ± 4
100	11 ± 2	5 ± 3	18 ± 2	110 ± 13	13 ± 1
333	8 ± 2	3 ± 1	17 ± 2	106 ± 17	10 ± 4
1000	10 ± 1	4 ± 1	17 ± 7	136 ± 11	14 ± 7
3330	10 ± 6 s	3 ± 1 m	22 ± 4	141 ± 20 s	17 ± 4
5000 SP	7 ± 2 s	2 ± 2 m	10 ± 5 s	144 ± 13 m	13 ± 3
With S9-mix[1]					
positive control	198 ± 46	340 ± 94	454 ± 31	1039 ± 8	274 ± 31
solvent control	10 ± 3	4 ± 1	20 ± 6	116 ± 4	15 ± 1
33	11 ± 2	4 ± 1	25 ± 8	119 ± 10	14 ± 3
100	10 ± 3	3 ± 2	30 ± 5	116 ± 4	16 ± 2
333	15 ± 2	4 ± 2	17 ± 5	133 ± 13	15 ± 1
1000	11 ± 2	4 ± 1	21 ± 2	167 ± 20	17 ± 3
3330	6 ± 4 s	3 ± 1 m	19 ± 3	129 ± 5 s	16 ± 4
5000 SP	8 ± 1 m	2 ± 1 m	15 ± 2	118 ± 21 m	14 ± 2

Solvent control: 0.1 ml dimethyl sulfoxide
1 The S9-mix contained 10% (v/v) S9 fraction
s Bacterial background lawn slightly reduced
m Bacterial background lawn moderately reduced
SP Slight Precipitate

APPENDIX I SUPPORTING MATERIALS AND METHOD

Bacterial background lawn evaluation

The condition of the bacterial background lawn is evaluated (if indicated), both macroscopically and microscopically by using a dissecting microscope (results are normal unless indicated in tables).

Definition	Characteristics
Normal	Distinguished by a healthy microcolony lawn.
Slightly reduced	Distinguished by a slight thinning of the microcolony lawn.
Moderately reduced	Distinguished by a moderate thinning of the microcolony lawn.
Extremely reduced	Distinguished by an extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
Absent	Distinguished by a complete lack of any microcolony background lawn.

Precipitation evaluation

Evidence of test article precipitate on the plates is recorded by addition of the following precipitation definition.

Definition	Characteristics
Slight Precipitate	Distinguished by noticeable precipitate on the plate. However, the precipitate does not influence automated counting of the plate.
Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, requiring the plate to be hand counted.
Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the required hand count difficult.

Evaluation of the reduction in the number of revertants

The reduction in the number of revertant colonies compared to number of revertants in the solvent control is evaluated as follows:
A reduction of 21-40%: slight reduction.
A reduction of 41-60%: moderate reduction.
A reduction of 61-99%: extreme reduction.
If the size of the microcolonies was increased to small colonies due to an extremely reduced background lawn the reduction is evaluated as microcolonies. If no revertant colonies are observed on the plates the reduction is evaluated as a complete lack of revertants.
However, any mean plate count equal to the minimal value of the historical control data range should be considered not toxic.

APPENDIX II DETAILED TABLES

Individual plate counts; (following pages)

Experiment 1
Strain TA1535

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1098	1170	1135	1134 ±	36
solvent control	15	12	17	15 ±	3
10	15	17	13	15 ±	2
33	14	17	20	17 ±	3
100	22	24	10	19 ±	8
333	16	22	16	18 ±	3
1000	18	16	19	18 ±	2

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	233	191	197	207 ±	23
solvent control	20	12	20	17 ±	5
10	12	14	15	14 ±	2
33	10	8	12	10 ±	2
100	18	21	17	19 ±	2
333	12	17	18	16 ±	3
1000	13	18	15	15 ±	3

APPENDIX II — continued —

Experiment 1
Strain TA1537

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	305	321	350	325 ±	23
solvent control	9	13	7	10 ±	3
10	13	7	9	10 ±	3
33	6	11	11	9 ±	3
100	8	10	10	9 ±	1
333	11	9	8	9 ±	2
1000	9	9	9	9 ±	0

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	415	406	342	388 ±	40
solvent control	10	7	7	8 ±	2
10	7	16	10	11 ±	5
33	9	9	4	7 ±	3
100	12	11	8	10 ±	2
333	9	12	8	10 ±	2
1000	6	7	7	7 ±	1

APPENDIX II — continued —

Experiment 1
Strain TA98

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1128	1135	1127	1130 ±	4
solvent control	32	26	27	28 ±	3
10	29	40	26	32 ±	7
33	33	28	36	32 ±	4
100	27	32	30	30 ±	3
333	34	35	29	33 ±	3
1000	31	26	34	30 ±	4

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	769	704	611	695 ±	79
solvent control	45	41	38	41 ±	4
10	31	36	29	32 ±	4
33	32	33	43	36 ±	6
100	35	25	39	33 ±	7
333	32	41	34	36 ±	5
1000	38	37	32	36 ±	3

APPENDIX II — continued —

Experiment 1
Strain TA100

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1323	1344	1338	1335 ±	11
solvent control	166	183	184	178 ±	10
3	175	186	195	185 ±	10
10	177	174	172	174 ±	3
33	177	192	198	189 ±	11
100	170	177	176	174 ±	4
333	201	207	180	196 ±	14
1000	189	208	184	194 ±	13
3330 s	185	193	182	187 ±	6
5000 m SP	162	199	222	194 ±	30

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1259	1303	1256	1273 ±	26
solvent control	171	181	189	180 ±	9
3	186	190	168	181 ±	12
10	177	200	162	180 ±	19
33	167	195	204	189 ±	19
100	164	193	169	175 ±	16
333	205	195	196	199 ±	6
1000	175	219	208	201 ±	23
3330 s	161	172	192	175 ±	16
5000 m SP	204	184	224	204 ±	20

s: Bacterial background lawn slightly reduced
m: Bacterial background lawn moderately reduced
SP: Slight Precipitate

APPENDIX II — continued —

Experiment 1
Strain WP_2uvrA

		WITHOUT S9-MIX				
plate		1	2	3	MEAN	SD
dose (µg/plate)						
positive control		793	744	721	753 ±	37
solvent control		17	14	23	18 ±	5
3		13	14	15	14 ±	1
10		15	8	14	12 ±	4
33		12	17	11	13 ±	3
100		11	9	10	10 ±	1
333		13	14	19	15 ±	3
1000		19	14	8	14 ±	6
3330		17	10	10	12 ±	4
5000	SP	19	19	16	18 ±	2

		WITH S9-MIX				
plate		1	2	3	MEAN	SD
dose (µg/plate)						
positive control		275	300	301	292 ±	15
solvent control		14	12	19	15 ±	4
3		18	14	19	17 ±	3
10		14	17	18	16 ±	2
33		14	15	10	13 ±	3
100		12	15	14	14 ±	2
333		14	13	11	13 ±	2
1000		24	24	23	24 ±	1
3330		13	14	17	15 ±	2
5000	SP	21	18	14	18 ±	4

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 2
Strain TA1535

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	973	1008	995	992 ±	18
solvent control	6	14	10	10 ±	4
1000	7	10	9	9 ±	2
3330 s	9	4	9	7 ±	3
5000 s SP	5	9	7	7 ±	2

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	206	213	182	200 ±	16
solvent control	10	11	16	12 ±	3
1000	7	12	12	10 ±	3
3330 s	6	6	10	7 ±	2
5000 m SP	8	11	8	9 ±	2

s: Bacterial background lawn slightly reduced
m: Bacterial background lawn moderately reduced
SP: Slight Precipitate

APPENDIX II — continued —

Experiment 2
Strain TA1537

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	494	487	723	568	± 134
solvent control	3	4	8	5	± 3
1000	5	10	9	8	± 3
3330 m	3	5	5	4	± 1
5000 m SP	3	0	2	2	± 2

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	265	432	450	382	± 102
solvent control	8	9	13	10	± 3
1000	4	9	6	6	± 3
3330 s	4	5	5	5	± 1
5000 m SP	3	5	6	5	± 2

s: Bacterial background lawn slightly reduced
m: Bacterial background lawn moderately reduced
SP: Slight Precipitate

APPENDIX II — continued —

Experiment 2
Strain TA98

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1115	1066	1193	1125 ±	64
solvent control	23	30	23	25 ±	4
1000	19	24	21	21 ±	3
3330	18	21	19	19 ±	2
5000 s SP	11	22	16	16 ±	6

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	665	675	667	669 ±	5
solvent control	17	18	29	21 ±	7
1000	18	24	21	21 ±	3
3330	19	14	19	17 ±	3
5000 SP	12	17	20	16 ±	4

s: Bacterial background lawn slightly reduced
SP: Slight Precipitate

APPENDIX II — continued —

Experiment 3
Strain TA1535

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1012	1006	1049	1022 ±	23
solvent control	4	9	7	7 ±	3
33	8	9	3	7 ±	3
100	10	13	11	11 ±	2
333	7	8	10	8 ±	2
1000	11	9	10	10 ±	1
3330 s	6	17	8	10 ±	6
5000 s SP	5	8	7	7 ±	2

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	160	250	185	198 ±	46
solvent control	8	14	9	10 ±	3
33	10	13	11	11 ±	2
100	6	12	12	10 ±	3
333	14	14	17	15 ±	2
1000	13	9	10	11 ±	2
3330 s	3	5	11	6 ±	4
5000 m SP	7	8	9	8 ±	1

s: Bacterial background lawn slightly reduced
m: Bacterial background lawn moderately reduced
SP: Slight Precipitate

APPENDIX II — continued —

Experiment 3
Strain TA1537

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	236	253	199	229 ±	28
solvent control	3	4	3	3 ±	1
33	7	3	6	5 ±	2
100	4	8	3	5 ±	3
333	3	2	4	3 ±	1
1000	4	5	3	4 ±	1
3330 m	2	4	3	3 ±	1
5000 m SP	0	2	3	2 ±	2

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	250	334	437	340 ±	94
solvent control	3	5	3	4 ±	1
33	5	4	3	4 ±	1
100	3	2	5	3 ±	2
333	6	4	2	4 ±	2
1000	5	4	4	4 ±	1
3330 m	3	4	3	3 ±	1
5000 m SP	3	2	2	2 ±	1

m: Bacterial background lawn moderately reduced
SP: Slight Precipitate

APPENDIX II — continued —

Experiment 3
Strain TA98

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1086	1153	1110	1116 ±	34
solvent control	15	25	21	20 ±	5
33	28	25	21	25 ±	4
100	17	17	20	18 ±	2
333	15	19	18	17 ±	2
1000	19	23	10	17 ±	7
3330	21	19	26	22 ±	4
5000 s SP	9	6	15	10 ±	5

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	431	489	442	454 ±	31
solvent control	14	24	23	20 ±	6
33	18	24	33	25 ±	8
100	35	25	29	30 ±	5
333	15	23	14	17 ±	5
1000	20	21	23	21 ±	2
3330	17	18	23	19 ±	3
5000 SP	13	15	16	15 ±	2

s: Bacterial background lawn slightly reduced
SP: Slight Precipitate

APPENDIX II — continued —

Experiment 3
Strain TA100

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1468	1414	1539	1474 ±	63
solvent control	110	119	122	117 ±	6
33	109	104	114	109 ±	5
100	125	105	100	110 ±	13
333	122	106	89	106 ±	17
1000	137	146	125	136 ±	11
3330 s	135	163	124	141 ±	20
5000 m SP	155	130	147	144 ±	13

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1044	1030	1042	1039 ±	8
solvent control	112	115	120	116 ±	4
33	116	111	130	119 ±	10
100	112	116	120	116 ±	4
333	146	120	133	133 ±	13
1000	182	175	144	167 ±	20
3330 s	129	124	133	129 ±	5
5000 m	99	140	116	118 ±	21

s: Bacterial background lawn slightly reduced
m: Bacterial background lawn moderately reduced
SP: Slight Precipitate

APPENDIX II — continued —

Experiment 3
Strain WP$_2$uvrA

		WITHOUT S9-MIX				
plate		1	2	3	MEAN	SD
dose (µg/plate)						
positive control		783	805	843	810 ±	30
solvent control		13	14	21	16 ±	4
33		14	18	21	18 ±	4
100		12	14	12	13 ±	1
333		7	14	8	10 ±	4
1000		13	22	8	14 ±	7
3330		17	13	20	17 ±	4
5000	SP	10	15	15	13 ±	3

		WITH S9-MIX				
plate		1	2	3	MEAN	SD
dose (µg/plate)						
positive control		305	244	272	274 ±	31
solvent control		14	15	16	15 ±	1
33		11	13	17	14 ±	3
100		14	17	17	16 ±	2
333		15	16	15	15 ±	1
1000		16	15	21	17 ±	3
3330		13	20	16	16 ±	4
5000	SP	12	14	15	14 ±	2

SP: Slight Precipitate